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14. ABSTRACT

The task of identifying novel and significant ER stress related changes that are regulated by ubiquitin ligases was initiated with a focus on Siah1/2, which led to a distinct understanding of these ligases' impact on melanoma, a study which continues. We have also identified the ubiquitin ligase RNF5, as one that regulates the immune checkpoint control mechanism, an ER stress associated ligase. We continue with exciting and significant studies as we define unexpected ER-stress related roles for the ubiquitin ligase RNF5 in melanoma. Lastly, our unbiased search for ubiquitin ligases that may affect melanoma resistance to BRAF inhibitor therapy led us to identify and characterize RNF125, which is downregulated in resistant tumors with concomitant increase in JAK1 and receptor tyrosine kinases. These important findings were published in *Cell Reports* (2015) and pave a path for new clinical trial for patients with resistant melanoma to BRAF inhibitors. We expect that the work with our current subset of Siah1/2 inhibitors will be summarized for publication during 2016/7.

15. SUBJECT TERMS

Siah1, Siah2, UPR, UBL, RNF5, RNF125, ubiquitin ligases, ER stress, JAK1, EGFR, melanoma, BRAF inhibitor resistance

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1. INTRODUCTION

The finding that Siah1/2 ubiquitin ligases expression may define a select cluster of melanoma led us to explore the precise role of each of these ubiquitin ligases in melanoma. Our studies allow for the first time to map the network of Siah1 and Siah2 regulated genes, revealing that these two family members represent distinct networks and are associated with different melanoma tumors. Given the role of Siah1/2 in control of the UPR and ER stress, as well as in the control of hypoxia, understanding mechanisms underlying each of these two ligases activity in melanoma is of crucial importance and will be continued during the second year of this funding. Parallel work on the ER stress associated ubiquitin ligase RNF5 (component of the ER associated degradation), led us to discover that it is directly involved in the regulation of immune checkpoint pathways. The significance of this finding is reflected in the limited growth (~30–40% of what is seen in wild type (WT) animals) of aggressive melanoma (Braf/Pten/Cdkn2a) when inoculated in RNF5 knockout (KO) animals. Significant increase in CD4 and CD8 positive T cells in the melanoma grown in RNF5 KO mice support a major role for this ubiquitin ligase on immune checkpoint control. In addition, we performed an unbiased screen to identify ubiquitin ligases that may be involved in the regulation of melanoma resistance to vemurafenib, a major obstacle in clinical management of melanoma today. Our search led to identify RNF125, which we found to regulate JAK1 stability with concomitant effect on EGFR and other receptor tyrosine kinases. The immediate implication of the funding of this project pertains to the possibility that JAK1 inhibitors may be used in clinical trials to defeat the resistance of such melanomas, an aspect that is currently being evaluated. Common to all 3 ubiquitin ligases is their intimate tie with ER stress and the direct implications to distinct aspects of melanoma development, progression and resistance. The second year of this grant has been devoted to (i) defining mechanisms underlying the role of Siah1/2 ubiquitin ligase in melanoma (ii) advancing the search as well as the characterization of Siah1/2 inhibitors (iii) defining the role of RNF5, a ubiquitin ligase which coordinates immune checkpoint and gut microbiota activity which defines degree of melanoma development.

2. KEYWORDS

Siah1, Siah2, UPR, UBL, RNF5, RNF125, ubiquitin ligases, ER stress, JAK1, EGFR, melanoma, BRAF inhibitor resistance.

3. ACCOMPLISHMENTS

What were the major goals of the project?
The major goals of this project as stated in the approved SOW are as follows:

Specific Aim 1 – Establish the significance of the Siah2–hypoxia–ER stress regulatory axis in melanoma development and response to chemotherapy of a subset of melanoma
Major Task 1: Determine the unique characteristics of melanomas that harbor the SHE gene signature; months 1–5
Major Task 2: Examine the role of Siah1/2 in regulating the ER stress and hypoxia responses in melanomas that do and do not exhibit the SHE gene expression signature; months 3–12
Major Task 3: Determine the biological significance of the ATF4–Siah2 component of the SHE regulatory axis to key melanoma phenotypes in cultured and xenograft melanoma models; months 3–12
Milestone 1*: Will have refined the components along the ER stress and hypoxia pathways, contributing to melanoma growth and response to therapy in cluster of melanomas; months 1–18*

Specific Aim 2 – Determine the effect of inhibitors of the Siah2–ER stress axis on melanoma development and response to chemotherapy

Major Task 1: Determine the effect of Siah1/2 inhibitors in melanoma cultures *in vitro*; months 6–18
Major Task 2: Determine the effect of Siah1/2 inhibitors in melanoma xenografts *in vivo*; months 8–24
Milestone 2*: Will have identified a novel approach to prevent and possibly overcome the resistance of melanoma to existing therapies*

Major Task 3: Determine the effect of Siah1/2 and ER stress inhibitors on melanoma development in genetic models, which recapitulates sun exposure of young age; months 8–24

Milestone 3*: Will have defined the ability of ER stress and Siah inhibitors to impact sun-induced melanoma.*

What was accomplished under these goals?

Specific Aim 1. Establish the significance of the Siah2–hypoxia–ER stress regulatory axis in melanoma development and response to chemotherapy of a subset of melanoma
Major Task 1: Determine the unique characteristics of melanomas that harbor the SHE gene signature (months 1–5). ***This task has been completed****.*

We completed a comprehensive mapping of Siah1 and Siah2 in melanoma, which for the first time revealed that each of these ligases has a distinct network of downstream targets and is expressed in different clusters of melanoma tumors. As shown in Figure 1, we mapped the signaling networks that are associated with Siah1 and with Siah2—revealing that two ubiquitin ligases are responsible for the regulation of different cellular networks. As shown in Figure 2, we mapped melanomas that express Siah1 (shown) or Siah2 (not shown) allowing us to further explore the significance and biological implications of each of these ubiquitin ligases for respective cluster of melanomas.

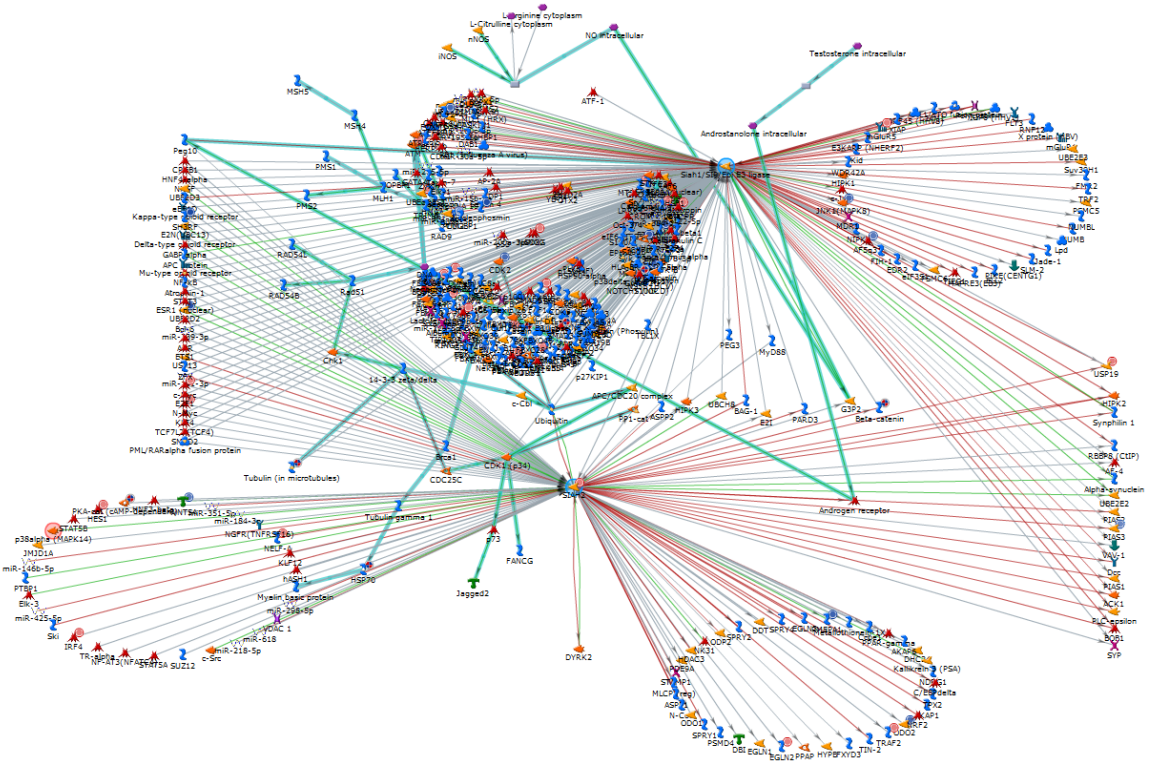


Figure 1*.* ***Mapping Siah1 and Siah2 networks in melanoma****. The summary depicts exhaustive characterization of Siah1 and Siah2 regulatory pathways that were mapped based on collection of datasets that were studied. Each of these links, whether direct or secondary, has clear implications for the biological effect of these two ubiquitin ligases on key cellular networks in melanoma.*

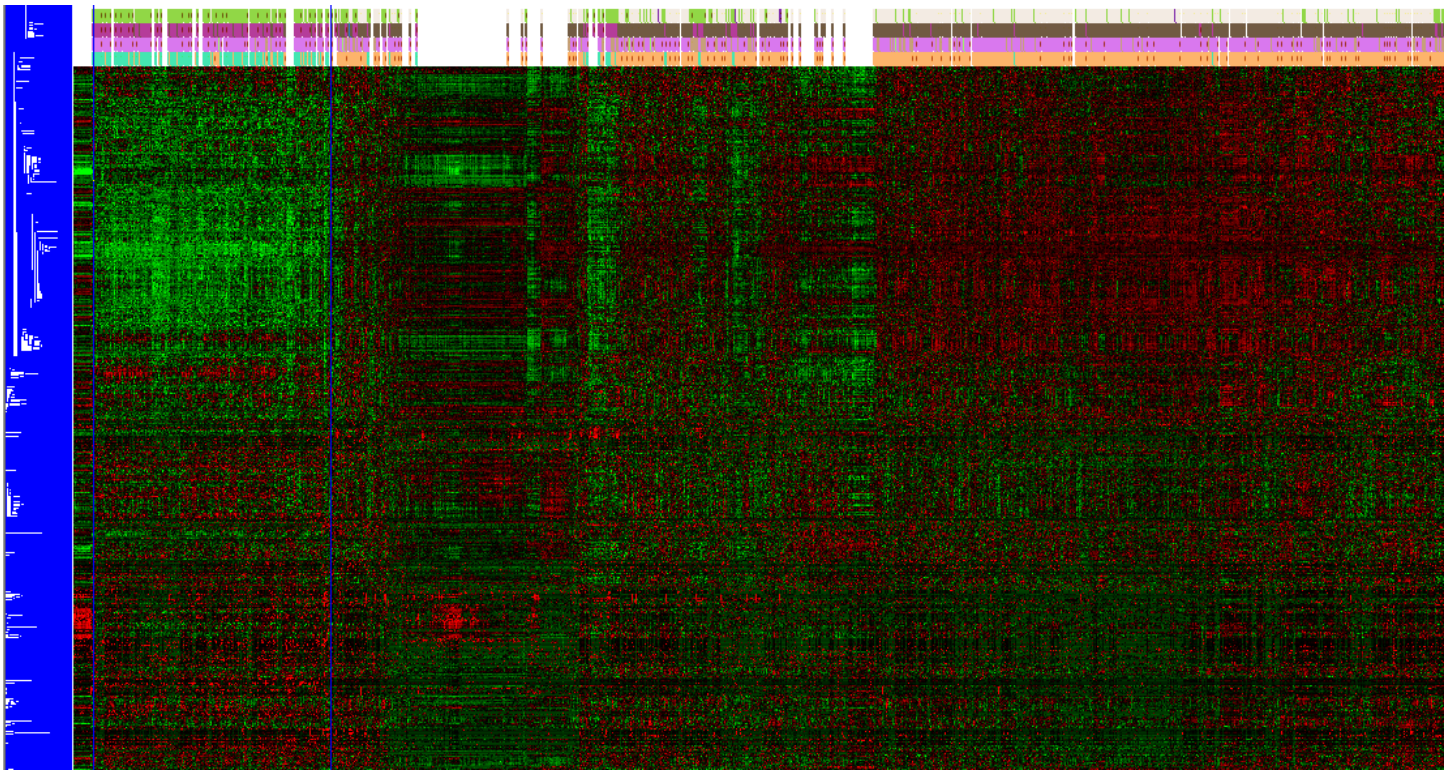


Figure 2. *Expression of Siah1 in melanoma establishes a clear subset that is positive for Siah1 expression (upper left green square). Similarly, a distinct subset of melanoma was identified as Siah2 expressing tumors.*

Major Task 2: Examine the role of Siah1/2 in regulating the ER stress and hypoxia responses in melanomas that do and do not exhibit the SHE gene expression signature (months 3–12). *Given the technical challenges that prevented us from completing the desired comprehensive assessment we wished to perform this task was not completed*.

We assessed the role of Siah1 and Siah2 in the distinct cluster of melanomas. This led us to the unexpected discovery that Siah1 on its own consists of three splice variants, which are expressed to different degrees in melanoma. We thus needed to determine the differences among the three splice variants and identify which among them is the most significant in melanoma. Among these splice variants we identified Siah1L to be expressed in a way that is best linked to the resistant phenotype of melanoma.

Following a comprehensive set of experiments we identified that Siah1L is linked to the ER stress by its effect on both ATF4 and on PGC1α, master regulator of mitochondrial biogenesis. The focus on Siah1L has advanced our understanding of Siah in melanoma and revealed an unexpected layer in the regulation of ER stress and mitochondrial biology, which is currently being explored.

During the second year of funding, we expanded our assessment of additional cell lines that were selected for analysis. Due to the complexity of the Siah variants (different splice forms), the number of cell lines we needed to assess was much greater than what we have in our laboratory (we have over 40 melanoma cell lines). While we assessed a much larger set of melanoma lines from our collaborators—Drs. Bosenberg and Halaban at Yale University and Dr. Herlyn at the Wistar Institute—we recognized the complexity in selecting cultures for further assessment, given that the splice variants of Siah1/2 are not easily detected, therefore, making it most difficult to identify upfront which of the many melanoma lines should be selected for analysis. On the top of these limitations, none of these splice variants is exclusively expressed, thus, making it even more difficult to link a specific phenotype seen in melanoma with select splice variant. These limitations are not specific for Siah splice variants, and are often appreciated in other genes who are subjected to processing, which

yields in multiple forms that are often co-expressed. We encountered this problem in another recent study conducted in our lab of the transcription factor ATF2, as detailed in a recent publication in *Cell Reports* (PMCID 27210757).

To complete this task, additional methods that are not currently available are needed, to define the repertoire of spliced variants expressed in tumors, and to further assess their relative contribution to a given phenotype.

Recognizing these obstacles, we devoted more efforts to the studies outlined below.

Major Task 3: Determine the biological significance of the ATF4–Siah2 component of the SHE regulatory axis to key melanoma phenotypes in cultured and xenograft melanoma models (months 3–12). ***This task will be completed in 2017, after the funds from the DoD have been exhausted***.

The discovery of Siah1 isoforms and the extensive studies we undertook to define their role in melanoma, relative to Siah2, delayed the *in vivo* experiments outlined in this task. As described above in Major Task 2, the technical obstacles prevented us from advancing this part of our studies, and yet, prompted us to advance parallel and related studies with ATF4, Siah and RNF5.

In context of Siah2, we discovered that *in vivo* inoculation of melanoma cells that were KD for Siah2 resulted in their slow development, within the first few days, compared with melanoma in which Siah2 was unaffected (Fig 3). Inoculation of lower numbers of melanoma cells further delayed melanoma development and enriched infiltration of the immune cells (Fig 3). This led us to test the possibility that an immune checkpoint mechanism may limit melanoma development, in its initial phase, in the absence of Siah2. Indeed, we confirmed a mark increase in the infiltration of immune cells to the melanoma which lacks Siah2, compared with the Siah2 WT expressing tumors (Fig 3). These observations point to the role of immune surveillance in this process, an aspect we are further exploring.

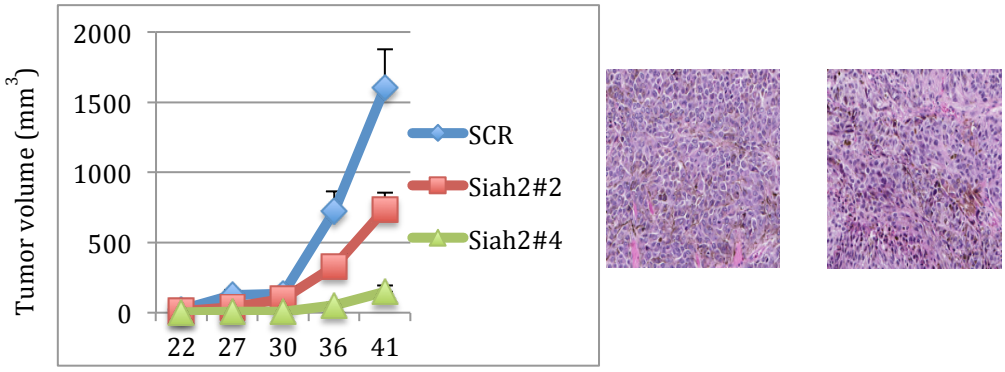


Figure 3. *Mouse NRAS mutant melanoma cells were stably infected with scrambled shRNA, or siah2#2 shRNA, or siah2#4 shRNA. After infection, 100,000 cells were injected s.c. on the flank of C56BL6 congenic mice. Tumor growth was monitored as noted. Right panel depicts infiltration of immune cells in the sh-Siah2 tumors.*

This interesting finding led us to further explore possible mechanisms that could underlie the phenotype elicited by Siah2. To this end, we performed two exploratory studies, namely, a gene expression array to identify the network of genes that are distinctly expressed in the Siah2 KO vs. WT melanoma, and, LC/MS/MS to identify which of the melanoma proteins binds to Siah2. The gene expression study was performed using RNAseq, where we mapped select pathways enriched (and downregulated) upon Siah2 KD (Fig 4).

RNAseq to identify genes and pathways that are deregulated in the Siah2 KD melanomas, led us to identify interferon signaling and antigen presentation pathways as major upregulated, and FXR/RXR, LXR/RXR activation as major downregulated pathways (Fig 4). Those were confirmed in set of qPCR reactions, allowing us to set up studies in which we define the role of Siah2 in controlling these signaling pathways.

Pathways upregulated in siah2 shRNA:
Antigen presentation pathway
Interferon signaling
Activation of IRF by cytosolic pattern recognition receptor
Role of BRCA1 in DNA damage
RhoA signaling
Upstream regulators:
Ifnar
TRIM24
IRF7
STAT1
ACKR2
Top disease:
Infectious disease

Pathways downregulated in siah2 shRNA:
FXR/RXR, LXR/RXR activation
Acute phase response signaling
Coagulation system
Upstream regulators:
HNF1A
PPARA
Ciprofibrate
Top disease:
Metabolic disease

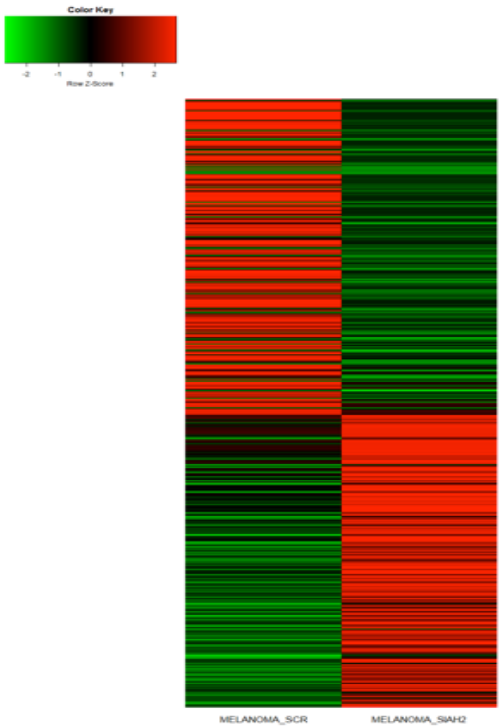


Figure 4. *Heat map of RNA-seq data from scrambled shRNA and siah2 shRNA melanomas, shown are top canonical pathways, upstream regulators and top diseases upregulated and downregulated in Siah2 shRNA melanoma. The selected genes have p-values less than or equal to 0.05 and fold-changes are greater than or equal to 2 (both directions). In the heatmap, the normalized expression signals are shown from green to red (lower signal to higher signal).*

To further understand the nature of Siah2 impact on melanoma, we performed an LC/MS/MS, where we identified Siah2 interacting proteins in melanoma tumors. This analysis led us to identify a number of proteins that are linked to interferon signaling. Among possible regulators of diverse IFN pathway, are RNA and DNA sensors, which may serve as upstream regulators of diverse IFN signaling. We characterized Zbp1 among a few and putative Siah2 substrates, for their possible regulation by this ubiquitin ligase. (Fig 5).

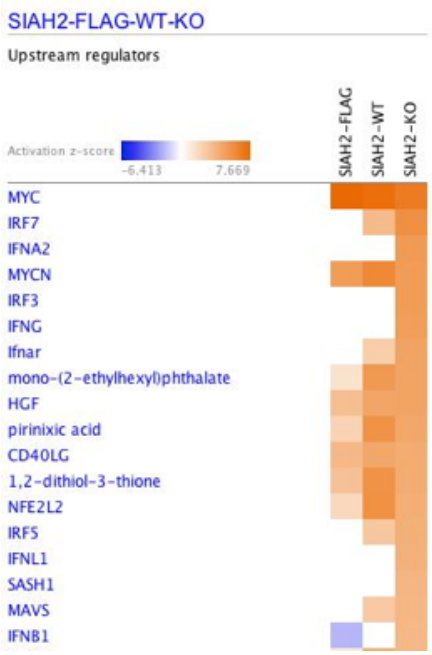


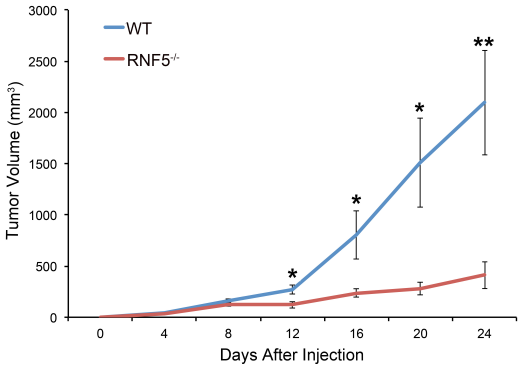
Figure 5. *The MS pathway analysis showed upstream regulators. Siah2 Ring Mutant (RM)-flag or empty vector-flag (EV) were overexpressed in 293T cells and immunoprecipitated with Flag beads. One aliquot of the immunoprecipitated Siah2RM-flag was used to perform MS (siah2-Flag). The other two aliquotes of Siah2 RM-flag or EV bound to beads were incubated with melanoma lysates from scrambled shRNA (Siah2-WT) or Siah2 shRNA, and Siah2-flag was analyzed by MS. EV was subtracted in all samples analyzed.*

Ongoing experiments, which we expect to complete during 2017, are confirming the new pathways found to be affected by Siah2, based on both gene expression and proteomics analyses. It appears that these studies may have uncovered novel regulatory cues that are Siah2-regulated, pathways that were never linked with Siah2 before, nor were they shown to be important in melanomagenesis. Thus, the novelty of our studies is expected to be significant to the field, although this will be completed after DoD funds are exhausted.

We conducted independent studies in collaboration with Dr. Colin Goding of Oxford, which led us to discover the role of ATF4 in melanoma metastasis. This powerful study defines ATF4 as a driver of melanoma migration and metastatic propensity, fueled by harsh environmental conditions associated with melanoma metastasis (lack of nutrients and hypoxic tension). This collaboration resulted in a study that is currently in press in the prestigious journal *Genes and Development* (listed in publication list below).

Parallel studies with another ubiquitin ligase that is part of the ERAD, RNF5, led us to discover that it plays a key role in immune checkpoint control, which are reflected in the slow growth of tumors of the BRAF/PTEN/CDKN2A mutant genotypes in the RNF5 KO animals. Here we found that the growth of congenic melanoma tumor cells is attenuated in RNF5 KO mice (Fig 6). This observation was confirmed by a number of congenic lines, including B16F10, YUMM1.3 and YUMM1.5.

***Figure 6**. Melanoma cells obtained from C57BL6 mice (Braf^{V600E}::Pten$^{-/-}$::Cdkn2a$^{-/-}$) were inoculated (700,000 cells) in B6 mice of WT and Rnf5$^{-/-}$ genotypes. Tumor growth was monitored at the indicated time points.*



We identified infiltration of CD4$^+$, CD8$^+$ and dendritic cells in the tumors, and that inactivation by neutralizing antibodies against CD4 or CD8 cells suffice to restore tumor growth, pointing to the inactivation of immune checkpoint(s) in RNF5 KO mice, enabling the limited growth of melanoma in these mice (Fig 7). Additional changes were identified in dentritic cells that were enriched in the tumors of the RNF5 KO mice, as well as cytokines that are produced in TILs of the RNF5 KO mice, including TNFa, IFNg and IL6 (not shown), clearly indicating an immune checkpoint phenotype in the absence of the RNF5 gene.

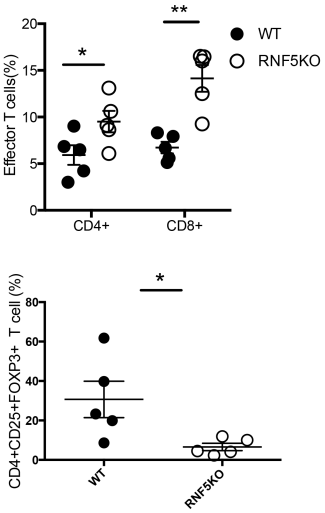


***Figure 7. Increased CD4 and CD8 positive TIL in tumors grown in RNF5 KO mice**. FACS was performed at 16 and 24 days (shown is 24 day time point) after inoculation of tumor cells in the WT and RNF5 KO mice, and number of CD8 positive and CD4 positive cells was determined. Likewise, the number of CD25-FOXP3-CD4 cells was determined, reflecting on the negative regulatory T cells whose presence has decreased in the tumors grown in the RNF5 KO mice.*

Further, recent reports point to the role of gut microbiota in the response to immune checkpoint therapy, therefore we monitored the effect of antibiotic administration or the effect of co-housing our *Rnf5*$^{-/-}$ mice with WT animals. In both cases, we observed a loss of the immune checkpoint phenotype, which coincided with restored melanoma growth (Fig 8). These results suggest that the gut microbiota may be involved in the control of immune checkpoint and melanoma development.

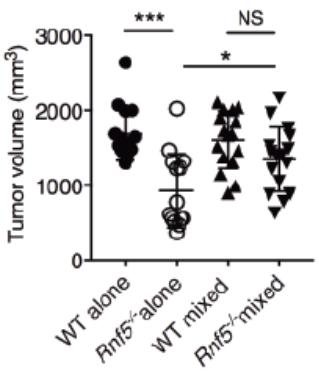


***Figure 8.** Mice of the indicated genotypes were co-housed for two weeks prior to tumor inoculation and the monitoring degree of tumor growth. Shown are data for tumor volume, 24 days after tumor cell inoculation.*

Ongoing studies are devoted to mapping of the microbiota of the RNF5 mutant compared with the WT genotypes. These studies identify distinct taxa that are enriched in the RNF5 KO gut microbiome, suggesting that such populations may be responsible for the changes identified in the immune response and growth of these tumors.

Since RNF5 is an ubiquitin ligase that is involved in ER stress and ERAD mechanisms, we monitored possible changes in ER stress in the intestinal epithelial cells. Notably, a marked increase was identified in the expression of both BIP and CHOP, in the *Rnf5*$^{-/-}$ intestinal cells. This finding points to the possible role of altered ER stress in the regulation of gut microbiota composition, an aspect that is currently being further assessed.

Of notable surprise was the finding that illi from the small intestine of *Rnf5*$^{-/-}$ animals exhibited a marked decrease in their length (Fig 9). Coincided with reduced villi size is the increase in the depth of crypts of these mice. Since altered villi length is usually associated with inflammation, the possibility that *Rnf5*$^{-/-}$ animals contain intrinsic inflammation was assessed.

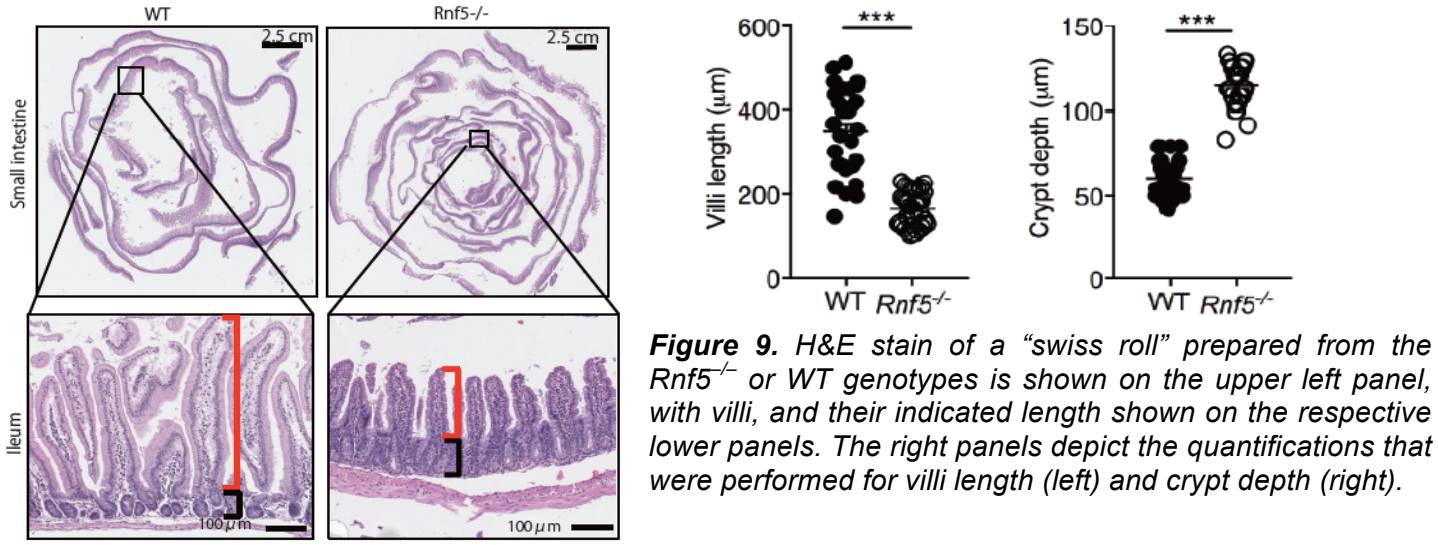


Figure 9. *H&E stain of a "swiss roll" prepared from the Rnf5*$^{-/-}$ *or WT genotypes is shown on the upper left panel, with villi, and their indicated length shown on the respective lower panels. The right panels depict the quantifications that were performed for villi length (left) and crypt depth (right).*

Ongoing studies are deciphering the mechanism underlying the cross talk between the immune system and the gut microbiota, as well as defining the specific taxons that are altered in RNF5 KO mice, and which are impacting the immune system. We expect to complete these studies during the course of 2017, with the subsequent publication acknowledging the support of this grant.

In a third project that was performed under the goal of defining ubiquitin ligase role in critical melanoma biology, we identified RNF125 as an ubiquitin ligase that is downregulated in tumors that developed resistance to therapy (BRAFi). We mapped the mechanism underlying RNF125 function in the resistant tumors, and identified JAK1 as its substrate. Further, RNF125–JAK1 was found to affect the expression of RTK, including EGFR, PDGFR and AXL, which were shown to be important in melanoma resistance phenotypes. This study was published in *Cell Reports* (2015, listed below), and is being considered for clinical trial, as we have shown that inhibition of JAK1 will help attenuate the resistance of melanoma to Vemurafenib.

Milestone 1: Will have refined the components along the ER stress and hypoxia pathways, contributing to melanoma growth and response to therapy in cluster of melanomas; (months 1–18). ***This task has been completed.***

We completed this milestone beyond original expectations as reflected in our current understanding of Siah1 and its isoforms, and Siah2, distinct function and respective melanoma cluster in which these are expressed. Added to this is our work on ER stress and RNF5, which identifies an unexpected role of this UBL in immune checkpoint control, and the discovery and characterization of RNF125 in melanoma resistance to vemurafenib.

Specific Aim 2. Determine the effect of inhibitors of the Siah2–ER stress axis on melanoma development and response to chemotherapy

Major Task 1: Determine the effect of Siah1/2 inhibitors in melanoma cultures ***in vitro*** (months 6–18). ***This task will be completed in 2017, after the funds from the DoD have been exhausted***.

Substantial effort was devoted to develop a class of Siah inhibitors that would be amenable for *in vivo* evaluation, which is not a trivial task. We approached this by performing two parallel tasks. In the first, we performed a screen for Siah1/2 small molecule inhibitors, using two independent approaches. Using AS/MS approach we screened 32,000 compounds for those that exhibit tight association with Siah1, association that can be identified by MS. Of those we identified beutolinic acid as one putative inhibitor, which was evaluated, in culture and *in vivo*. Analogs were synthesized and assessed in these line of studies as well. Figure 10 outlines the effect of these compounds on the growth of melanoma cells in culture, using 2D and 3D assays.

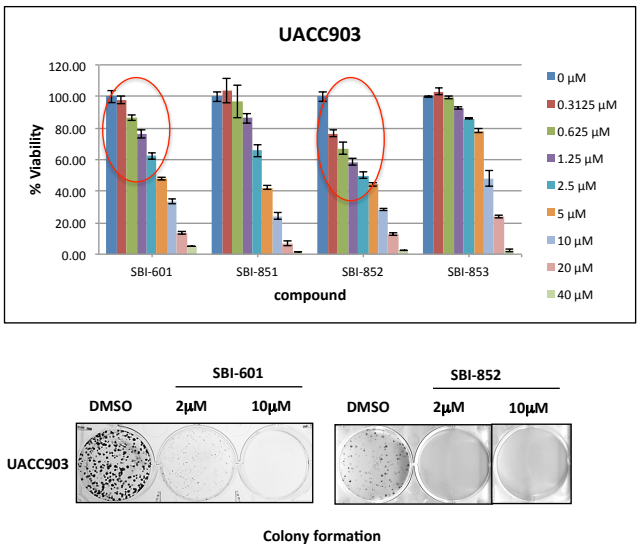
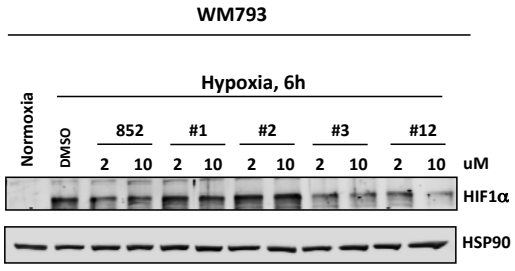


Figure 10. Characterization of Siah1/2 small molecule inhibitors*. A dose-dependent evaluation of SBI-601 and SBI-852 performed on a series of melanoma cells demonstrates dose dependent inhibition of melanoma cell growth in 2D (upper) and 3D (lower) culture conditions (shown is representative line, UACC903).*

Additionally, we performed a high-throughput screen for small molecules that could affect Siah1/2 confirmation, and hence activity. Over 32,000 compounds were screened using a thermal shift assay, a sensitive method that identifies the change in the melting temperature of a protein, which is expected to occur upon tight association with a small molecule. Of 17 positive hits identified from this screen, a series of secondary and tertiary assays were carried out to verify activity of the select small molecules as Siah inhibitors. Three of the 17 compounds (#1, #3, and #12) were confirmed to exhibit inhibition of Siah ubiquitin ligases. As shown in Figure 11, each of these new compounds effectively inhibits the level of HIF1α, a surrogate marker for Siah1/2 activity.

Figure 11. Small molecule inhibitors of Siah1/2 attenuate the expression level of HIF1α. *Indicated concentrations of the Siah1/2 inhibitory small molecules were added to WM793 melanoma cells for 24 h before cells were harvested and assessed for the level of HIF1α protein. Since Siah1/2 is causing the degradation of prolyl hydroxylase 1/3, a negative regulator of HIF1α, inhibition of Siah2 is expected to increase the expression of PHD3 with a concomitant decrease in the level of HIF1α.*



Further, evaluation of these compounds in 3D growth, which were monitored for colony forming efficiency or their ability to grow on soft agar, identified compounds #1, #3, and #12 to exhibit the most significant effect (Fig 12, left panel). Likewise, assessment of these compounds in a soft agar growth of melanoma cells confirmed their effective inhibition of the cell's growth in semisolid medium (Fig 12**,** right panel). Notably, SBI-852 was used as a positive control in these assays, demonstrating its strong inhibition of Siah, and respectively, of HIF1α and CFE of melanoma cells (Fig 10 and 11). The differences in the degree of inhibition among the melanoma cell lines and the distinct inhibitors, is noted.

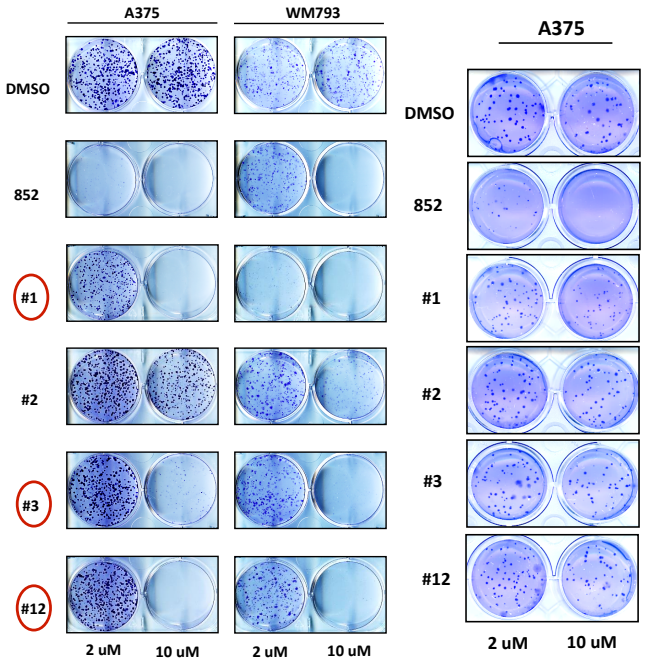


Figure 12. Select small molecules inhibitors of Siah1/2 effectively attenuate the 3D growth of melanoma cells. *Indicated melanoma cell lines were subjected to 3D growth in culture, where the effect of indicated small molecules was assessed. Five hundred cells plated were assessed 10 days later by crystal violet staining, demonstrating the dose-dependent inhibition of the melanoma growth by these inhibitors.*

In parallel to the characterization of SBI-852, we performed an independent screen to identify possibly Siah inhibitors based on their ability to cause a change in the conformation of Siah1 protein. Such changes can be assessed in high throughput using the thermal shift assay, which measures at high sensitivity the changes in protein confirmation upon interaction with bound compounds. Using a truncated form of Siah1, which was produced in bacteria, we screened a 32,000 compound library, leading to the identification of 80 compounds that altered Siah1 activity. Of those, 12 were further assessed for their ability to inhibit Siah activity, using HIF1α as a surrogate marker. Of those, 3 were identified to possess such capacity (not shown) and were further assessed for their ability to affect Siah1 ubiquitination *in vitro*. None of these compounds were able to impact the degree of Siah auto-ubiquitination (not shown), we then set to repeat this screen using a full length Siah1 protein. Using insect cells, a full length Siah1 protein was produced and purified, and confirmed for its ubiquitin ligase activity (not shown). Using the full length Siah1 protein, we repeated the screen with the initial 80 hits identified in the former thermal shift assay, and a library of additional 600 compounds of known drugs were used for the thermal shift assay screen. Of these, we selected 15 compounds for further assessment in culture, evaluating their ability to inhibit HIF1α stability. Of the 15 compounds, 11 exhibited the most remarkable inhibition of HIF1α expression (Fig 13a). The structure of compound 11 is of adapalene, a small molecule consisting of retinoic acid moiety (Fig 13b,c), which has been shown to possess antitumor activities in culture and *in vivo.* To further asses the properties of adapalene, we performed a dose-dependent inhibition of 12 melanoma cell lines, consisting of human and mouse melanomas. Across these melanoma cultures, a dose of 1–5 μM was effective in causing 50% or more death. Notably, a slightly higher dose (5 μM) was sufficient to cause cell death in a vemurafenib-resistant melanoma (Fig 13d), consistent with previous reports that point to the effective toxicity of this compound in different tumor cells. Using a colony forming assay, we assessed the effect of adapalene on 5 melanoma cultures, including the vemurafenib resistant cells. This analysis confirmed the effective inhibition of CFE of each of these melanoma cultures, including the drug resistant one (Fig 13e).

Given the impressive effect of adapalene, we have set to available analogues, which allow to define critical moieties required for its activity. Of eight such analogues tested, those that lacked or altered the retinoic acid moiety were no longer active, suggesting that it is required for the biological changes identified. Further, two of the eight compounds were found to exhibit at least as potent inhibition of HIF1α expression (not shown). Notably, one of these compounds exhibited a significantly (10-fold) more effective cell death on melanoma cultures (not shown). Ongoing work is devoted to the characterization of these newly identified small molecules both in culture and *in vivo*. We expect to complete this work during 2017.

Additionally, we advanced the development of a Siah inhibitory peptide, which we identified over the past couple of years and demonstrated its effectiveness in cultures of melanoma. We also developed

the ability to administer a new class of peptides that were modified to enable *in vivo* delivery by IV injection and are currently testing them in tumor models in mice. Development of such peptide took place in the course of the second year of funding and we expect to reach *in vivo* evaluation assays in the first half of 2017.

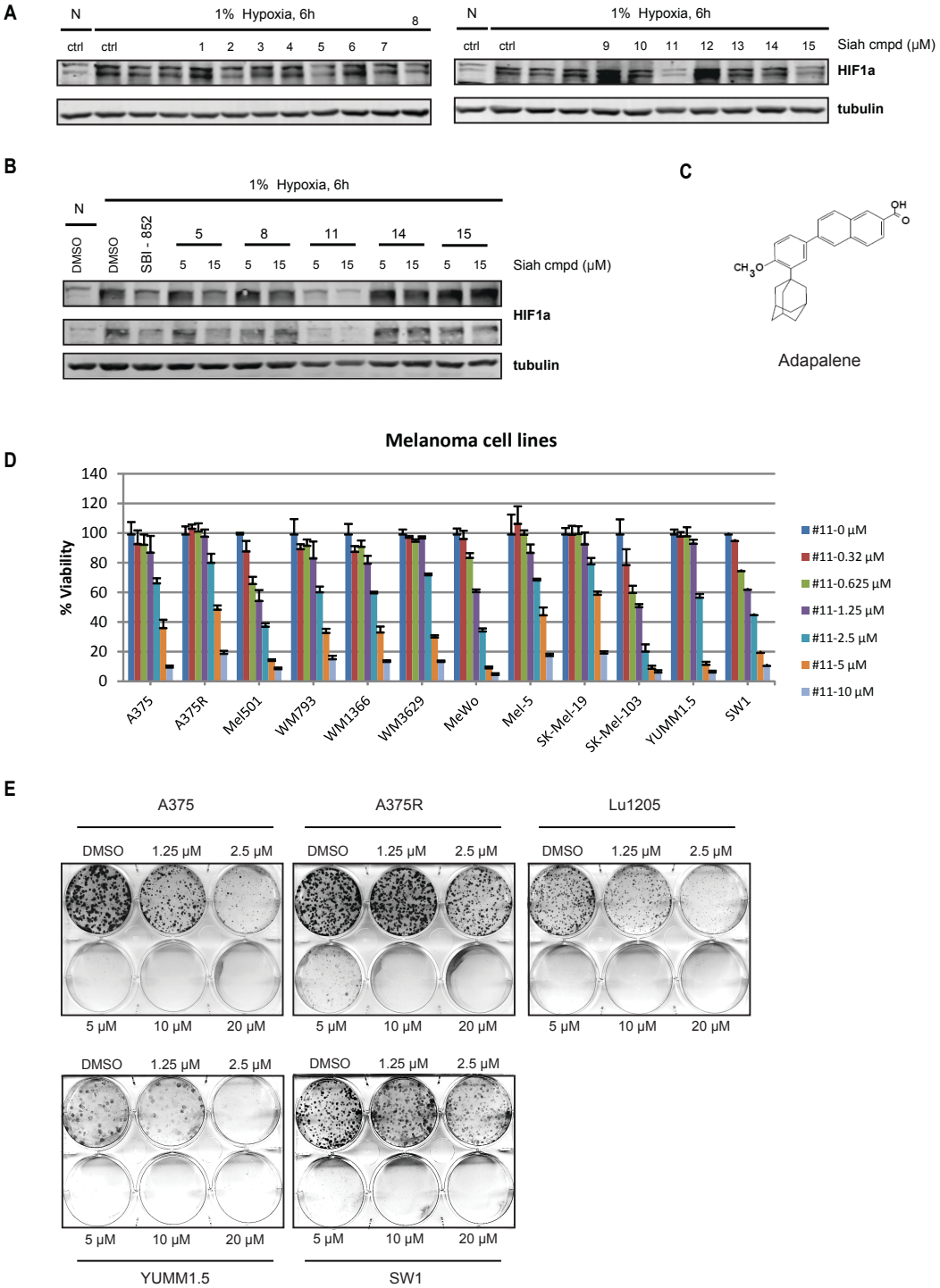


Figure 13. *(**A**) Inhibition of hypoxia-induced HIF1α induction by 15 selected compounds. Melanoma cells were incubated with either vehicle (DMSO) or indicated compounds (10 μM) for 6 h under hypoxia. Cells were harvested and whole cell lysates were immunoblotted with the indicated antibodies. (**B**) Compound #11 inhibited hypoxia-induced HIF1α induction in a dose dependent manner. (**C**) Structure of compound #11. (**D**) Different melanoma cells were plated in 96-well plates and incubated with the indicated concentrations of compound #11. Cell viability was assessed 72 h after treatment. (**E**) Indicated cultures were plated at low density (500 cells/well in 6-well plates) and grown in medium containing indicated concentration of compound #11. The number of colonies formed after 10 days in culture was determined by crystal violet staining.*

Major Task 2: Determine the effect of Siah1/2 inhibitors in melanoma xenografts *in vivo* (months 8–24).

Efforts were devoted to the *in vivo* evaluation of the first small molecule compound described above, SBI-852 (aka SBI-0646852). To evaluate effectiveness of SBI-852 *in vivo,* we first tested its bioavailability in mice. PK studies revealed good bioavailability at concentration of 5 or 25 mg/kg (Fig 14).

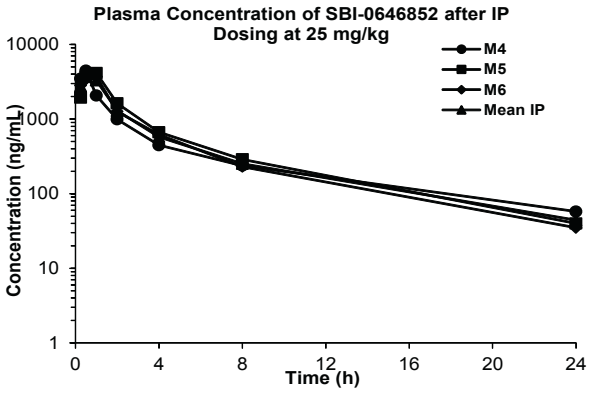
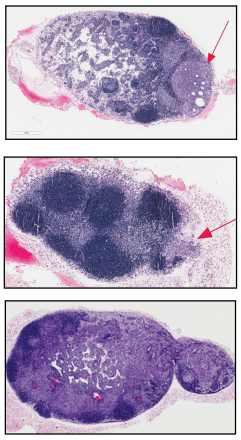


Figure 14. *In vivo PK study. SBI-852 was administered to fasted (C57/Bl/6J male) mice by IP injection (25 mg/kg). Plasma was collected at indicated time points and compound levels were measured by LC/MS/MS.*



Treatment	Mouse ID	Score
DMSO	1	+++
	2	++
	3	-
	4	-
	5	-
852-20mg/kg	1	-
	2	+
	3	-
	4	-
	5	-
852-40mg/kg	1	-
	2	-
	3	-
	4	+
	5	-

Based on the PK data, we performed *in vivo* studies to evaluate SBI-852's effect on the growth of melanoma YUMM1.3 cells. Unlike the data obtained in culture, *in vivo* SBI-852 did not exhibit any significant effect on the growth of the YUMM1.3 cells (not shown). Unlike the effect on tumorigenicity, the degree of lung metastasis was somewhat reduced in animals that were subjected to treatment by SIB-852 (Fig 15).

Figure 15. *Representative H&E staining of lymph nodes from mice bearing YUMM1.3 melanoma.*

Experiments using small molecule inhibitors took place in the course of the second year of this DoD funding. Additional inhibitors that were identified in the course of the second year will be evaluated in the first part of 2017, together with the Siah inhibitory peptide, which was further developed during the second year of funding, for *in vivo* effectiveness.

We limited our work for Siah inhibitors, using three different classes of Siah inhibitors that we identified and characterized during the period supported by this DoD funding.

Milestone 2: Will have identified a novel approach to prevent and possibly overcome the resistance of melanoma to existing therapies.

We advanced this milestone by virtue of the development of first-in-class inhibitors for ubiquitin ligases, which reached evaluation *in vivo*, and by the advance in developing small molecule inhibitors for Siah using a state of the art approach. Characterization of newly identified inhibitors initiated during the second year will commence during the last year of studies. Small molecules that appeared to be most promising in culture did not exhibit such activities *in vivo*. The option to further develop analogues of proper capabilities to deliver such activities *in vivo* is among our current considerations, while we have advanced development of new small molecules which are currently being evaluated. This will, ultimately, allow us to establish novel therapeutic modality for treatment of cancer, focused on melanoma.

Major Task 3: Determine the effect of Siah1/2 and ER stress inhibitors on melanoma development in genetic models, which recapitulates sun exposure of young age (months 8–24).

Milestone 3: Will have defined the ability of ER stress and Siah inhibitors to impact sun-induced melanoma.

Given the delay in the identification of a potent inhibitor, as outlined above, we delayed the performance of this experiment. We expect to carry it out, during 2017, using one of the peptides identified to be potent Siah2 inhibitor.

What opportunities for training and professional development have the project provided?

The following are the opportunities for training and professional development that this project has provided:

The Sanford Burnham Prebys Medical Discovery Institute (SBP) Office of Education, Training & International Services (OETIS) oversees and coordinates an annual individual development planning (IDP) process for all postdocs at the Institute. The focus of the IDP process at SBP is the career goal of the postdoc; identification of what skills, knowledge, and accomplishments will be necessary for the postdoc to obtain a desired independent position following training; and identification of training and professional development opportunities that are available for the postdoc to obtain the necessary skills and knowledge. The SBP OETIS provides guidance and advising to both postdocs and PIs throughout the postdoc's training with respect to developing IDPs and preparing for a successful transition to independence post-training. The SBP OETIS also maintains webpages containing comprehensive resources on career path identification, career planning, and creating an IDP that can be utilized in conjunction with the formal annual IDP process. Dr. Yan Li, a postdoctoral trainee, is directly supported by this grant and works on the RNF5 project and is part of the active training program.

The SBP IDP process includes two components:

1. **First-Year IDP.** Within the first 3 months of beginning postdoctoral training at SBP, all postdocs receive and fill out an initial "planning and expectations" document to discuss with their PI. This document serves as the foundation for their postdoctoral IDP and is designed to facilitate discussion between the PI and new postdoc regarding goals and expectations for the first year of training, as well as stimulate initial discussions about long-term career goals and training plans.
2. **Postdoctoral IDP.** At the end of the first year of training SBP postdocs receive notification that it is time to update their IDP, and they receive the information they included in their first-year planning and expectations document in the form of a full IDP that they can update with their accomplishments over the past year and their goals for the coming year, mid-term future, and long-term future. Each subsequent year of their postdoctoral training, postdocs will receive notification and the previous year's IDP form to update and expand. The IDP forms are designed to build upon each previous year as well as provide a solid foundation from which a postdoc can easily build his or her CV/resume.

The individual(s) listed in section 7 of this report began their postdoctoral training at SBP and have participated in the SBP annual postdoctoral IDP process described above.

How were the results disseminated to the communities of interest?

2015: The results described in this report were presented at multiple international ubiquitin conferences, which were held in June 2015 in China and in September 2015 in Dobrovnik Croatia. The work was also presented in June 2015 in Iceland at a melanoma workshop. To the greater communities these results were disseminated through OSHER program at UCSD, in April 2015.

2016: The results described in this report are consistently updated on Dr. Ronai's laboratory web page, ronailab.net. In addition, the results of these studies were discussed in each of the following conferences that were attended by Dr. Ronai in 2016: European Melanoma Workshop (Veranna, Italy; June 2016), SMR (Boston, MA; Nov. 2016), Masonic Cancer Center (University of Minnesota, Minneapolis; March 2016), 4th International Conference on Tumor Microenvironment and Cellular Stress (Rhodes, Greece; June 2016), Signgene Symposium (Berlin, Germany; Sept. 2016), European Society for Pigment Cell Research (Milano, Italy; Sept. 2016), Centre National de la Recherche Scientifque (Institut Curie, Paris, France; Sept. 2016).

What do you plan to do during the next reporting period to accomplish the goals?

We expect to complete the tasks indicated in our proposal as detailed above. Despite the extensive work performed, we were not able to complete some of the tasks either due to unforeseen technical difficulties (Splice forms) or poor *in vivo* activity (some of the Siah inhibitors). We expect to complete the studies with small molecule inhibitors, as well as Siah inhibitory peptide, during 2017. Likewise, we expect to complete our studies with RNF5 for publication in 2017 and to initiate a clinical trial with MGH (led by Dr. Keith Flaherty, Yale) in early 2017.

4. IMPACT

What was the impact on the development of the principal disciplines of the project?

The mapping of Siah ubiquitin ligases to distinct regulatory networks in a select set of melanomas enables a new level of refinement for these ubiquitin ligases.

Identifying the role of Siah ubiquiting ligase in immune checkpoint, and hence in the growth of melanoma, offers new insight into the regulation of these important pathways by Siah ligases.

Defining the pathways that are regulated by Siah2 in melanoma will advance our understanding of melanoma development and identify new players and their regulation.

The development of small molecule inhibitors to ubiquitin ligases is a novel unprecedented opportunity to tackle one of the most warrented nodes known to play significant role in cancer, and in this particular case, Siah in melanoma.

What was the impact to other disciplines?

The identification of RNF5–ER Stress UBL as a player in immune checkpoint and the implications for melanoma points to the first UBL, that has been mapped to the immune checkpoint control.

Finding how RNF5 impacts gut microbiota composition offers new insight into the coordination of immune checkpoint and gut microboiome activity by a ubiquitin ligase.

What was the impact on technology transfer?

Finding JAK1 as a novel target for treatment of melanoma with BRAF inhibitor resistance, based on our studies with RNF125, is being evaluated in a clinical trial that will open in 2017.

What was the impact on society beyond science and technology?

The ability to develop peptides that specifically inhibit Siah ubiquitin ligase *in vivo* offers a new paradigm for targeting ubiquitin ligases *in vivo*. The understanding of RNF5 coordination of immune

checkpoint and gut microbiome offers a new paradigm for understanding how these fundamental processes are linked.

5. CHANGES/PROBLEMS

Changes in approach and reasons for change.

The splicing of variants for Siah and the difficulties in their detection, as the complexity in their assessment had forced us to change course in our proposed studies.

Actual or anticipated problems or delays and actions or plans to resolve them.

The activity of Siah inhibitory peptides and small molecules did not pan out *in vivo*, as seen in culture. This led us to initiate new screening campaigns that delayed final assessment of our multiple screening initiatives. Our concerted efforts resulted in the identification of new small molecules, and advancing our inhibitory peptide to a form that can be tested *in vivo*. We expect to complete the first line of key experiments in early 2017.

Changes that have a significant impact on expenditures.

Additional expenses incurred due to the new screening campaigns, and development of new inhibitory peptides will be covered by independent funding sources.

6. PRODUCTS

Publications, conference papers, and presentations

2015: Kim H, Frederick DT, Levesque MP, Cooper ZA, Feng Y, Krepler C, Brill L, Samuels Y, Hayward NK, Perlina A, Piris A, Zhang T, Halaban R, Herlyn MM, Brown KM, Wargo JA, Dummer R, Flaherty KT, Ronai ZA. Downregulation of the Ubiquitin Ligase RNF125 Underlies Resistance of Melanoma Cells to BRAF Inhibitors via JAK1 Deregulation. *Cell Rep*. 2015 Jun 9;11(9):1458-73.

2016: Senft, D., Ronai, ZA. Adaptive Stress Responses During Tumor Metastasis and Dormancy. *Trends in Cancer.* 2016 August;2(8):429-442. PubMed PMID: 27868104.

2017:Falletta, P., Sanchez-del-Campo, L., Chauhan, J., Effern, M., Kenyon, M., Kershaw, CK., Siddaway, R., Lisle, R., Freter, R., Daniels, MJ., Lu, X., Tüting, T., Middleton, M., Buffa, FM., Willis, AE., Pavitt, G., Ronai, ZA., Sauka-Spengler, T., Hölzel, M., Goding, CR. Translation reprogramming is an evolutionarily conserved driver of phenotypic plasticity and therapeutic resistance in melanoma. *Genes and Development*, In Press.

Li, Y., Tinoco, R., Elmén, L., Feng, Y., Fujita, Y., Kim, H., Yooseph, S., Zarecki, R., Khateb, A., Tam, MA., Ruppin, E., Peterson, SN., Bradley, LN., Ronai, ZA. Coordinated regulation of gut microbiota and immune checkpoint activity by the ubiquitin ligase RNF5 determines melanoma development. Under revision.

Feng, YM., Scortegagna M., Ronai, ZA. Characterization of novel Siah1/2 inhibitors in melanoma. To be submitted by April 2017

Conference presentations

2015: UB workshop, China, June 2015
Melanoma Workshop, Croatia, June 2015
Ubiquitin Meeting, Croatia, September 2015

2016: European Melanoma Workshop, Veranna, Italy, June 2016
Masonic Cancer Center, University of Minnesota, Minneapolis, March 2016
4th International Conference on Tumor Microenvironment and Cellular Stress, Rhodes, Greece, June 2016
Signgene Symposium, Berlin, Germany, Sept. 2016
European Society for Pigment Cell Research, Milano, Italy, Sept. 2016
Centre National de la Recherche Scientifque, Institut Curie, Paris, France, Sept. 2016

Website(s) or other Internet site(s)

ronailab.net

Technologies or techniques

Nothing to report.

Inventions, patent applications, and/or licenses

Nothing to report.

Other products

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Ze'ev Ronai
Project Role:	Principal Investigator
Researcher Identifier:	
Nearest person month worked:	1
Contributions to Project:	PI; oversaw the development and progress of the project and coordinated the collaboration with a number of national and international groups.
Funding Support:	N/A

Name:	Marzia Scortegagna
Project Role:	Staff Scientist
Researcher Identifier:	
Nearest person month worked:	2
Contributions to Project:	Performed experiments related to Siah and RNF125.
Funding Support:	N/A

Name:	Yan Li
Project Role:	Postdoctoral Fellow
Researcher Identifier:	

Nearest person month worked:	5
Contributions to Project:	Performed experiments related to RNF5.
Funding Support:	N/A

Name:	Yongmei Feng
Project Role:	Staff Scientist
Researcher Identifier:	
Nearest person month worked:	1
Contributions to Project:	Developed the exhaustive bioinformatics-based assessment of Siah.
Funding Support:	N/A

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

The following is a description of active support changes since the submission of this grant:

Grants that have ended:

R01 CA188372-01 (PI: Ronai, Z.) 04/01/15–05/31/20 1.20 calendar (10%)
NIH/NCI $228,750
Understanding and Targeting the Glutamine Carrier SLC1A5 in Breast Cancer
Goals: The major goal of this project is to advance the understanding of Gin metabolism in BCa and provide the foundation for novel stratification methods and therapeutic modalities for BCa.
Specific Aims: (1) Identify RNF5-dependent and –independent transcriptional, translational, and post-translational events regulating SLC1A5/38A2 availability and activity in representative BCa cultures. (2) Establish the biological significance of SLC1A5/38A2 expression in BCa cells for cellular metabolism, autophagy, growth, and response to therapy. (3) Using BCa tumor samples, we will determine the relationship between BCa expression of SLC1A5/38A2 and RNF5, the response to treatment, and disease outcome.
Scientific Officer: Sarah M. Lee
National Institutes of Health
9000 Rockville Pike
Bethesda, MD 20892
Phone: (240) 276-6280
E-mail: sarah.lee@nih.gov

R01 CA172017-03 (PI: Ronai, Z.) 07/01/13–04/30/18 1.2 calendar (10.0%)
NIH/NCI $207,500
ATF2 Oncogenic Addiction in Melanoma
Goals: The major goal of this project is to identify unrecognized pathways for melanoma development that could be exploited for the development of innovative therapeutic modalities.
Specific Aims: (1) Determine the mechanisms and significance of ATF2 addiction to PKC epsilon in melanoma. (2) Evaluate the oncogenic and tumor suppressor functions of ATF2 using conditional ATF2 knock-in mice. (3) Identify natural compounds that promote nuclear export of ATF2 in melanoma cells.
Grants Officer: Cammie La
National Institutes of Health
9000 Rockville Pike
Bethesda, MD 20892

Phone: (240) 276-6323
E-mail: cl311z@nih.gov

R01 CA179170-03 (PI: Ronai, Z.) 09/12/13–07/31/18 1.20 calendar (10%)
NIH/NCI $231,963
PDK1 as a Novel Target in Melanoma
Goals: The major goal of this project is to determine whether PDK1 is an important signaling node required for the most aggressive forms of prostate cancer.
Specific Aims: (1) Determine the role of PDK1 in Nras and Braf mutant melanomas harboring wild-type Pten. (2) Characterize the molecular mechanisms underlying PDK1 control of melanoma development and progression. (3) Identify biomarkers for PDK1-sensitive tumors and determine the clinical significance of PDK1 expression in melanoma.
Grants Officer: Barbara Fisher
National Institutes of Health
9000 Rockville Pike
Bethesda, MD 20892
Phone: (301) 631-3012
E-mail: bfisher@mail.nih.gov

Pending grants that are now active:

R35 CA197465-01 (PI: Ronai, Z.) 02/02/16–01/31/23 6.0 calendar (50%)
NIH/NCI $600,000
Rewired Signaling at the Nexus Melanoma Metastasis and Resistance
Goals: The major goal of this project is to establish novel mechanisms underlying tumor plasticity, enabling the development of novel agents for predicting, monitoring, and preventing tumor metastasis and resistance.
Scientific Review Officer: Michael B. Small
National Institutes of Health
9000 Rockville Pike
Bethesda, MD 20892
Phone: (240) 276-6438
E-mail: smallm@mail.nih.gov

P01 CA128814-06A1 (PI: Ronai, Z.) 05/05/16–04/30/18 1.72 calendar (14.3%)
NIH/NCI $1,101,098
ER Stress and Mitochondrial Biogenesis in Melanoma
Goals: The major goal of this project is to identify new therapeutic strategies for overcoming drug resistance and metastatic disease in melanoma.
Specific Aims: (1) Define the role of UPR components (Siah1 isoform 2, ATF4,CHOP) in melanoma metastasis and resistance to therapy. (2) Determine the role of proliferator-activated receptor gama coactivators (PGC-1a/b) and downstream signaling in melanoma and acquisition of drug resistance. (3) understand the role and regulation of a feedback loop between MITF and ATF4, and the resulting downstream metabolic alterations, in acquisition of the invasive and drug-resistant phenotype of melanoma.
Scientific Review Officer: Caterina Bianco
National Institutes of Health
9000 Rockville Pike
Bethesda, MD 20892
Phone: (240) 276-6459
E-mail: biancoc@mail.nih.gov

R01 CA202021 (PI: Ronai, Z.) 07/01/16–06/30/21 0.65 calendar (5.4%)
NIH/NCI $322,722
Control of Protein Synthesis by the UPS Under Stress
Goals: The major goal of this project is to establish the importance and significance of select components in a novel regulatory network that controls protein synthesis during cellular stress, and establish its role in BCa using a combination of BCa cultures and xenografts, RPPA technology, and TCGA dataset mining.
Specific Aims: (1) Establish the physiological significance of the RACK1-JNK-eEF1A2 regulatory axis to the cellular stress response, growth, and therapeutic response to BCa. (2) Assess the role of stress-induced polysomal recruitment of Nedd8-Cullin machinery in regulating the decay of newly synthesized proteins in BCa. (3) Determine the importance of UBQLN1 recruitment to polysomes in regulating newly synthesized proteins under stress conditions and in modulating the reponse of BCa to therapy.
Scientific Review Officer: Nywana Sizemore
National Institutes of Health
9000 Rockville Pike
Bethesda, MD 20892
Phone: (301) 408-9915
E-mail: sizemoren@csr.nih.gov

What other organizations were involved as partners?

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

Nothing to report.

9. APPENDIX

Nothing to report.